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Interaction of the Papillomavirus E8^E2C Protein with the Cellular CHD6 Protein Contributes to Transcriptional Repression▽†

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Expression of the E6 and E7 oncogenes of high-risk human papillomaviruses (HPV) is controlled by cellular transcription factors and by viral E2 and E8^E2C proteins, which are both derived from the HPV E2 gene. Both proteins bind to and repress the HPV E6/E7 promoter. Promoter inhibition has been suggested to be due to binding site competition with cellular transcription factors and to interactions of different cellular transcription modulators with the different amino termini of E2 and E8^E2C. We have now identified the cellular chromodomain helicase DNA binding domain 6 protein (CHD6) as a novel interactor with HPV31 E8^E2C by using yeast two-hybrid screening. Pull-down and coimmunoprecipitation assays indicate that CHD6 interacts with the HPV31 E8^E2C protein via the E2C domain. This interaction is conserved, as it occurs also with the E8^E2C proteins expressed by HPV16 and -18 and with the HPV31 E2 protein. Both RNA knockdown experiments and mutational analyses of the E2C domain suggest that binding of CHD6 to E8^E2C contributes to the transcriptional repression of the HPV E6/E7 oncogene promoter. We provide evidence that CHD6 is also involved in transcriptional repression but not activation by E2. Taken together our results indicate that the E2C domain not only mediates specific DNA binding but also has an additional role in transcriptional repression by recruitment of the CHD6 protein. This suggests that repression of the E6/E7 promoter by E2 and E8^E2C involves multiple interactions with host cell proteins through different protein domains.

Persistent infections with "high-risk" human papillomaviruses (HPV) are a major risk factor for cervical cancer development (5, 10, 86). Carcinogenic HPV encode the E6 and E7 oncoproteins, which are required for the immortalization of normal human keratinocytes and the continous growth of cervical cancer cell lines, such as HeLa (40, 41). Expression of E6 and E7 transcripts is controlled by both cellular and viral transcription factors (70). Papillomavirus proteins derived from the E2 gene are involved in control of viral transcription, of DNA replication, and of segregation of viral genomes (29, 37, 38). The E2 open reading frame gives rise to multiple gene products by alternative RNA splicing. In addition to the full-length E2 protein, cells infected with bovine papillomavirus type 1 (BPV1), cottontail rabbit papillomavirus (CRPV), and HPV types 1, 11, 16, 31, and 33 express an E8^E2C transcript in which the small E8 domain is fused to the C-terminal domain of E2 (E2C) (9, 18, 28, 46, 52, 58, 63). Inactivation of E2 in the HPV16 genome increases E6/E7 transcription (59). Mutation of E8^E2C in the HPV31 or HPV16 genome increases genome copy number and E6/E7 transcripts, strongly suggesting that transcriptional repression by E8^E2C plays an important role in viral replication (31, 63, 85).

The C-terminal domain (E2C) which is shared by E2 and E8^E2C is responsible for sequence-specific recognition of $ACCN_6GGT$ DNA sites (E2 binding site [E2BS]) and homo- and heterodimerization of E2 proteins (38). The amino terminus of E2 is involved in the activation of DNA replication, modulation of viral transcription, and genome segregation (26, 55, 56). This is mediated by the interaction of the amino-terminal domain of E2 with viral and cellular proteins. The activation of replication is mainly due to the interaction with the viral E1 protein (60). Binding of E2 to Brd4, ChlR1, and Mklp2 has been implicated in mediating the plasmid segregation function of E2 (4, 36, 47, 82). Transcriptional activation by E2 depends upon binding to several cellular proteins, such as Brd4, cNAP1, Gps2, p300, and TopBP1 (7, 16, 27, 39, 49). E2 has also been shown to repress the HPV E6/E7 promoter from promoter-proximal binding sites (70). Several reports have demonstrated that the E2C part derived from HPV11, -16, -18, or -31 alone has significant repression activity in transient-transfection experiments using HPV E6/E7 promoter constructs (11, 13, 17, 65). This phenomenon has been attributed to binding site competition between E2C and cellular transcription factors such as SP1 and TATA box binding protein (14, 17, 19, 69). However, it has been demonstrated that the amino termini of E2 and HPV31 E8^E2C (31E8^E2C) enhance the repression of the HPV E6/E7 promoter (22, 43, 48, 64, 65). For E2, this has been linked to the interaction of the amino terminus with the cellular Brd4, EP400, and SMCX

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proteins (57, 77, 79). In side-by-side comparisons, 31E8^E2C is a more potent repressor of the HPVE6/E7 promoter than E2 (63, 65). In contrast to E2, 31E8^E2C acts as a transcriptional repressor from both promoter-distal and -proximal E2BS (65). This is due to the presence of the E8 domain, which functionally interacts with transcriptional corepressors, such as HDAC3 and NCor1 (1, 48).

In order to find additional cofactors we conducted a yeast two-hybrid genetic screen with 31E8^E2C. Using this approach we identified the chromodomain helicase DNA binding domain 6 (CHD6) protein as an interactor with 31E8^E2C. CHD6 belongs to the CHD protein family, whose members are characterized by a highly conserved double chromodomain and a highly conserved SWI2/SNF2 ATPase/helicase domain (21, 54, 76). In contrast, the DNA binding domain is not conserved and might only be functional in the founding members, CHD1 and -2 (12, 61). Currently nine members have been identified in the human genome. CHD1 and -2 belong to subfamily 1, CHD3 and -4 to subfamily 2, and CHD5 to -9 form subfamily 3 (23). Genetic, biochemical, and structural analyses of CHD protein family members have suggested that they are transcriptional regulators and are involved in developmental processes (23, 35). Very little is currently known about CHD6, which is a ubiquitously expressed gene (34, 54). Mammalian CHD6 has been reported to reside in the nucleus and colocalize with RNA polymerase II (34). Consistent with a role in transcription control, CHD6 has been shown to interact with transcription factors such as the Nrf2 protein, which recognizes antioxidant response elements in promoters and activates transcription (42). CHD6 is a DNA-dependent ATPase and thus may have nucleosome remodeling activity, as has been suggested for human CHD3 (34, 74).

Our data suggest that CHD6 interacts with the E2C domain of E8^E2C proteins derived from HPV16, -18, and -31 and also of E2. RNA knockdown experiments and mutational analyses of the E2C domain indicate that binding of E8^E2C to CHD6 contributes to the transcriptional repression of the HPV E6/E7 promoter. This suggests that the E2C domain, in addition to its DNA binding activity, recruits cellular proteins that negatively regulate the HPV E6/E7 promoter.

MATERIALS AND METHODS

Plasmids. The luciferase reporter plasmids pC18-SP1-luc and pGL18URR-luc have been previously described (64, 65). Plasmid pC18-SP1-luc harbors four E2BS in front of a minimal promoter, and plasmid pGL18URR-luc contains the complete upstream regulatory region (URR) of HPV18. The expression plasmids for untagged and hemagglutinin (HA)-tagged 31E8^E2C (pSG 31 E8^E2C and pSG 31 E8^E2C-HA) have been previously described (64, 65). Mutations were introduced by overlap extension PCR and confirmed by DNA sequence analysis (GATC, Konstanz, Germany). MBP-E8^E2C was constructed using PCR with pSG31 E8^E2C as a template and primers MBP-E8-BamHI for (5′-CTCACTATAGGGCGGATCCATGGCA-3′) and pSG 1167 rev (5′-ACCA CAACTAGAATGCAGTGAA-3′). After BamHI digestion, the amplicon was inserted into the BamHI site of pMALc2X (New England Biolabs, Frankfurt am Main, Germany). MBP-31E8^E2C$_{1\text{-}91}$ and MBP-31E2C$_{53\text{-}184}$ were constructed by deleting a SphI/XbaI fragment or a ZraI/BamHI fragment, respectively, from MBP-31E8^E2C. MBP-31E8^E2C 118/119 was constructed by inserting a ZraI/BsrGI fragment from pSG 31 E8^E2C-HA-118/119 (64) into ZraI/BsrGI-digested MBP-31E8^E2C.

To generate pIRES-puro-31E8^E2C-HA and pIRES-neo-31E8^E2C-HA, the 31E8^E2C-HA coding sequence was amplified by PCR using primers E8 NheI for (5′-ACATTAGCTAGCGAATTCCATGGCAATAC-3′) and pSG 1167 rev. The amplicon was digested with NheI and BamHI and inserted into NheI/BamHI-digested pIRES-puro3 or pIRES-neo2 (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France).

The construction of expression vectors for HA-tagged versions of HPV16 and HPV18 E8^E2C (pSG 16E8^E2C-HA and pSG 18E8^E2C-HA) will be described in a future publication by Fertey et al. (unpublished data). pSX31 E2 and pSX 31E2 HA have previously been described (62, 64). We generated a mammalian expression vector for CHD6 by inserting the full-length human CHD6 cDNA (formerly CHD5) (54) into a KpnI/NotI-digested pcDNA5.1 FR/TO plasmid (Invitrogen); the integrity of the resulting vector, pcDNA5.1 FR/TO CHD6, was confirmed by sequencing the cDNA-plasmid insertion sites and by extensive restriction analysis. To generate pDrive CHD6, the amino acid (aa) 1 to 474 region (aa 1-474) of the RNA from HPV18-positive cells was amplified by reverse transcription-PCR (RT-PCR) using primers CHD6 F2 forward, 5′-TCT TCCTTCAATAGATGAAAATGAAA-3′, and 1420 reverse, 5′-TAGCTCCCA CGTGCTTTCTT-3′, and the amplicon was subcloned into pDrive (Qiagen, Hilden, Germany). MBP-CHD6 1-474 was generated by PCR using pDrive CHD6 aa 1-474 as a template and primers CHD6 in pMal 179 for 5′-ATGAA AATGAAAATACAGAAAA AAGAGAAGCAGTTG-3′ and CHD6 in pMal 1420rev 5′-ATATATATAAGCTTTGACTCCCACGTGCTTTC-3′. The amplicon was digested with HindIII and inserted into XmnI/HindIII-digested pMalc2X.

Yeast two-hybrid assay. Yeast two-hybrid screening was performed using GAL4 fusion proteins as previously described (20). Briefly, *Saccharomyces cerevisiae* strain AH109 (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) was transformed with the pGBKT7-31E8^E2C bait plasmid using the lithium acetate method and stably maintained in the absence of tryptophan. Yeast cells were subsequently transformed with a pACT-based cDNA library derived from Epstein-Barr virus-transformed human peripheral lymphocytes (75) and grown (10^6 primary transformants) on His/Leu/Trp dropout plates. His^+ colonies were tested for beta-galactosidase activity by using the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside filter assay. Prey plasmids from clones positive in both assays were isolated and propagated in *Escherichia coli* strain DH5α and sequenced using primers Gal4 rev 951 (5′-TCTTCAGACACTTGG CGCA-3′) and YADH for 686 (5′-CTGCACAATATTTCAAGCTATACC-3′).

MBP protein expression and pull-down assays. *Escherichia coli* BL21(DE3) pLysS Rosetta II cells carrying the respective MBP plasmids were grown to an optical density at 600 nm of 0.7, isopropyl-β-D-thiogalactopyranoside was added to a concentration of 0.5 mM, and cells were incubated for an additional 3 h at 20°C. Extracts were obtained by sonication of pelleted bacteria in 50 mM Tris (pH 7.5), 200 mM NaCl, 1 mM EDTA, 1 mM dithithreitol (DTT) with protease inhibitors (complete mini EDTA free; Roche Diagnostics, Mannheim, Germany). After the addition of Igepal 630 to a final concentration of 1%, the supernatant was clarified by centrifugation (15 min, 4°C, 12,000 rpm). MBP fusion proteins were purified by incubation with amylose resin (New England Biolabs, Frankfurt am Main, Germany), followed by extensive washing with 50 mM Tris (pH 7.5), 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% (vol/vol) Igepal 630, and protease inhibitors.

Similar amounts of MBP or MBP fusion proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining, were incubated for 2 h at 4°C with reticulocyte lysate containing *in vitro*-translated ^{35}S-labeled target proteins or whole-cell lysates. Beads were pelleted by centrifugation and washed several times with 1 ml of buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Igepal 630, 1 mM DTT, and protease inhibitors). Bound proteins were eluted with 4× SDS gel loading buffer (Carl Roth, Karlsruhe, Germany), heated to 95°C, and then separated by SDS-PAGE. ^{35}S-labeled proteins were detected by phosphorimaging of dried gels.

For pull-down experiments using whole-cell lysates, transfected or untransfected HeLa cells were lysed in buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 1 mM EDTA, 1 mM DTT, protease inhibitors, 0.5% Igepal 630) and cleared by centrifugation. Cell lysates were incubated with MBP-fusion proteins bound to amylose beads as described above. After extensive washing bound proteins were detected by immunoblotting with the respective antibodies. All pull-down experiments were repeated at least once with different MBP protein preparations to ensure reproducibility.

DNA-protein interaction assay. A double-stranded ^{32}P-labeled oligonucleotide (150,000 cpm) matching the HPV31 E2 binding site 4 (nucleotides 45 to 70; 5′-GTGGTGAACCGAAAACGGTTGGTATA-3′) was incubated with equal amounts of purified MBP-fusion proteins for 20 min in binding buffer (10 mM HEPES [pH 7.9], 125 mM NaCl, 1 mM EDTA, 5 mM DTT, 10% [vol/vol] glycerol, protease inhibitors) on ice, and then the beads were washed twice with binding buffer. The amount of retained oligonucleotide was determined by scintillation counting.

Coimmunoprecipitation. Cells were washed in phosphate-buffered saline (PBS) and lysed in buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1 mM DTT, protease inhibitors, 0.5% Igepal 630) for 15 min under gentle agitation at 4°C. The lysate was cleared by centrifugation (14,000 rpm at 4°C) and then incubated with anti-HA-coupled magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 1 h. Beads were then extensively washed in a μMACS column with lysis buffer. Bound proteins were eluted by addition of 30 μl of 4× SDS gel loading buffer and identified by immunoblotting (Carl Roth, Karlsruhe, Germany).

Cell culture. HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) supplemented with gentamicin and 10% fetal bovine serum (Seromed Biochrom, Berlin, Germany). RTS3b cells were maintained in E-medium containing 5% fetal bovine serum. For colony reduction assays, HeLa cells were transfected with the respective plasmids and Fugene HD (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

To generate shCHD6 cell lines, HeLa cells were infected with different lentiviral shRNA constructs directed against human CHD6 (no. 032221; Sigma-Aldrich, Germany) or with a control virus (shc001V; Sigma-Aldrich, Germany) according to the manufacturer's protocol and then selected for 12 to 14 days with 0.4 μg/ml puromycin. Pooled cultures were used for further analyses. For colony formation assays, 3×10^5 HeLa-shcontrol, HeLa-shCHD6-1, or HeLa-shCHD6-2 cells were transfected in 60-mm dishes with 1 μg of the respective expression vectors. One day after transfection, cells were split onto 100-mm dishes and 24 h later selected with 500 μg/ml G418. After 10 to 12 days of selection, colonies were washed with PBS, fixed with acetone-methanol (1:1; vol/vol) solution, stained with eosin solution, and then counted.

Stable 293T cells were generated by transfecting 1 μg pIRESpuro-31E8^E2C-HA or pIRESpuro3 plasmid. The cells were selected with 0.4 μg/ml puromycin, and resistant colonies were pooled for further analyses.

Luciferase reporter assays. Approximately 3×10^4 HeLa cells were seeded into 24-well dishes the day before transfection. Cells were cotransfected with 50 ng of luciferase reporter plasmids, 10 ng of the respective expression vector DNA, or empty vector pSG5 as indicated in the figure legends. Transfections were carried out using Fugene HD (Roche Diagnostics, Mannheim, Germany) and Opti-MEM (Invitrogen, Karlsruhe, Germany). Luciferase activity was determined 48 h posttransfection as previously described (65).

Immunoblot analyses. Transfected cells were lysed 48 h posttransfection in 30 μl of 4× SDS gel loading buffer (Carl Roth, Karlsruhe, Germany) heated to 95°C and separated by SDS-PAGE. Proteins were transferred in 10 mM cyclohexylaminopropane sulfonic acid (pH 10.3) on a nitrocellulose membrane (Protran; Whatman, Dassel, Germany). Membranes were blocked by incubation in Tris-buffered saline–0.1% Tween 20–5% nonfat dry milk for at least 30 min and then incubated with the diluted primary antibodies HA-probe (1:1,000; MMS-101P; Covance) anti CHD6 (1:100; sc-81066; Santa Cruz Biotechnology), anti-Trim28 (1:3,000; Transduction Laboratories), and anti-α-tubulin (1:1,500; CP06; Oncogene). Bound antibodies were detected with mouse antibodies conjugated to horseradish peroxidase (polyclonal rabbit anti-mouse immunoglobulin–horseradish peroxidase; 1:3,000; Dako) and Super-Signal West Dura reagent (Perbio Science, Bonn, Germany). Chemiluminescent signals were recorded with a FluorSMax imaging system and quantitated using the QuantityOne software (Bio-Rad, Munich, Germany).

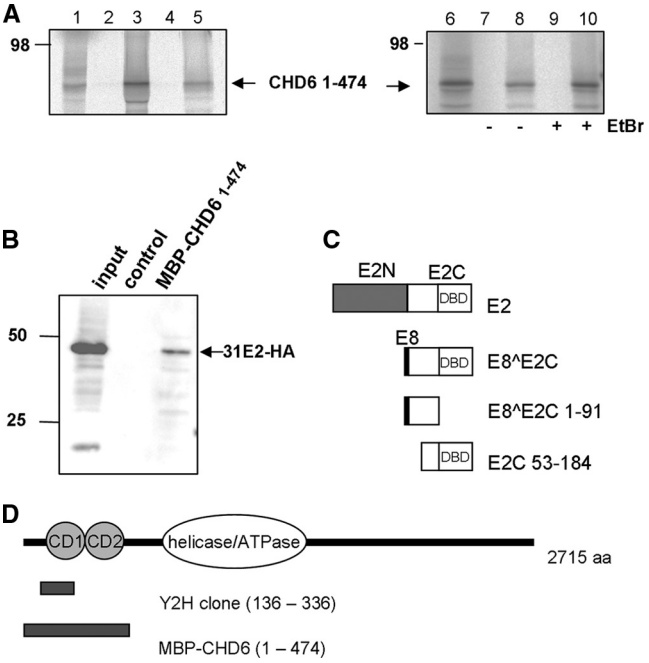

FIG. 1. The C-terminal domain of 31E8^E2C interacts with CHD6 fragments. (A) Similar amounts of bacterially expressed and purified MBP as a control (lanes 2, 7, and 9), MBP-31E8^E2C (lanes 3, 8, and 10), MBP-31E8^E2C$_{1-91}$ (lane 4), and MBP-31E2C$_{53-184}$ (lane 5) proteins were incubated with ^{35}S-labeled *in vitro*-translated CHD6 aa 1-474 fragment. In lanes 9 and 10 ethidium bromide (50 μg/ml) was added to the binding reaction mixture. Molecular size markers (in kDa) are indicated to the left. Samples were washed extensively, and then the retained proteins were separated by SDS-PAGE and analyzed by phosphorimaging. In lanes 1 and 6, 10% aliquots of the *in vitro* translation reaction mixtures are shown. (B) Similar amounts of bacterially expressed and purified MBP (control) or MBP-CHD6$_{1-474}$ fusion proteins were incubated with cell extracts derived from HeLa cells transfected with the expression vector for HA-tagged 31E2. After washing, bound proteins were analyzed by immunoblotting for the presence of E2-HA by using an HA antibody. The input represents an aliquot of the cell extract from transfected cells. On the left a molecular size marker (in kDa) is shown. (C) Structures of E2 and E8^E2C. E2 consists of the amino-terminal E2N and the E2C domain, which contains the DNA binding domain (DBD). In E8^E2C, E8 replaces the E2N. Also shown are deletion constructs used in the experiment shown in panel A, lanes 4 and 5. (D) Schematic structure of CHD6. Conserved chromodomains 1 (CD1) and 2 (CD2) as well as the conserved helicase/ATPase domain are indicated. Gray bars indicate the cDNA fragment found by yeast two-hybrid screening and the CHD6 fragment used for *in vitro* interaction studies.

RESULTS

The amino terminus of CHD6 interacts with the C-terminal domain of 31E8^E2C. To identify host cell factors interacting with the 31E8^E2C protein, we screened a cDNA library with full-length 31E8^E2C fused to the Gal4 DNA binding domain as a bait in a yeast two-hybrid assay. One of the putative interactors encoded a partial cDNA for CHD6 (residues 136 to 336), which belongs to the CHD family of proteins that have conserved structural motifs implied in chromatin remodeling and have been suggested to be involved in the control of transcription (35). To further investigate the interaction of 31E8^E2C with CHD6, a partial cDNA of CHD6 (residues 1 to 474) encompassing the region identified in the Y2H screen was cloned from human keratinocytes immortalized by the HPV18 genome (30). The truncated CHD6 protein contains the double chromodomain (aa 292 to 439) but lacks the ATPase/helicase domain. To verify the interaction *in vitro*, bacterially expressed, purified MBP-31E8^E2C was incubated with *in vitro*-translated, ^{35}S-labeled CHD6$_{1-474}$ (Fig. 1A). Only MBP-31E8^E2C, and not MBP alone, bound to CHD6$_{1-474}$, confirming an interaction between 31E8^E2C and CHD6 (Fig. 1A). The 31E8^E2C consists of three parts: the conserved E8 domain (aa 1 to 12), which has repression activity (65, 85), the nonconserved hinge region (aa 13 to 101), and the E2C terminus, which mediates DNA binding and dimerization (aa 102 to 184). To further map the interacting domain of 31E8^E2C, MBP-31E8^E2C$_{1-91}$ and MBP-

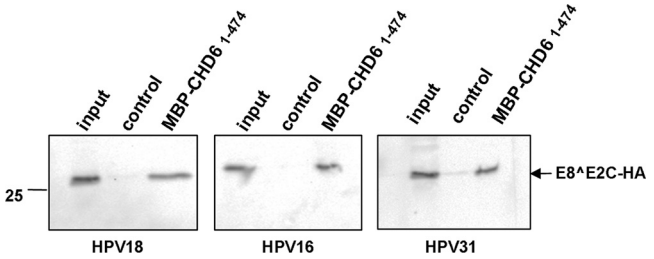

FIG. 2. Binding to CHD6 is conserved among different high-risk HPV types. Expression vectors for HA-tagged E8^E2C of HPV types 16, 18, and 31 were transfected in HeLa cells, and 48 h posttransfection whole-cell lysates were prepared. Similar amounts of bacterially expressed and purified MBP as a control and MBP-CHD6$_{1-474}$ were incubated with these cell extracts. After washing, bound proteins were analyzed by immunoblotting for the presence of E8^E2C-HA proteins with an antibody against HA.

31E2C$_{53-184}$ were tested for CHD6$_{1-474}$ binding. This revealed that the interaction occurred with the E2C domain but not with the E8 repression domain or the hinge domain (Fig. 1A). To analyze whether DNA binding activity is required for the E2C-CHD6 interaction, binding reactions were performed in the presence of 50 μg/ml ethidium bromide, which has been shown to disrupt DNA-protein interactions but not protein-protein interactions (32). The addition of ethidium bromide did not change the binding of E8^E2C to CHD6, suggesting that DNA binding is not required (Fig. 1A, lanes 8 and 10). This was further confirmed by using the DNA binding-deficient mutant E8^E2C 118/119 (see Fig. 4A and D). The interaction of E2C with CHD6 indicated that also the full-length E2 protein may interact with CHD6 via the E2C domain. In line with this, HPV31 E2-HA transiently expressed in HeLa cells bound to MBP-CHD6$_{1-474}$ but not MBP alone (Fig. 1B). This strongly suggested that CHD6 interacts with both E2 and E8^E2C via the E2C domain and that this is independent from the different amino-terminal domains.

The binding of E8^E2C to CHD6 is conserved among different high-risk papillomavirus types. To investigate whether the binding is conserved among high-risk HPV types, we additionally cloned transcripts encoding E8^E2C proteins of HPV types 16 and 18 (unpublished results). To facilitate detection of the respective proteins, an HA epitope was inserted in the hinge region, where it does not interfere with the function of 31E8^E2C or 31E2 (64). HeLa cells were transiently transfected with expression vectors for 16E8^E2C-HA, 18E8^E2C-HA, or 31E8^E2C-HA, and then whole-cell lysates were incubated with MBP-CHD6$_{1-474}$ or MBP. As shown in Fig. 2, the different E8^E2C proteins interacted with similar efficiencies with CHD6$_{1-474}$, suggesting that this interaction is conserved among high-risk HPV types.

We then evaluated whether the 31E8^E2C protein interacted with the full-length form of CHD6, which encodes a 2,715-aa protein. Whole-cell extracts derived from HeLa cells were incubated with the purified MBP-31E8^E2C protein and retained proteins were analyzed by immunoblotting with a CHD6 antibody. As can be seen in Fig. 3A (input, endogenous), the antibody recognizes a major band of ~300 kDa, consistent with the calculated molecular mass of 305 kDa for

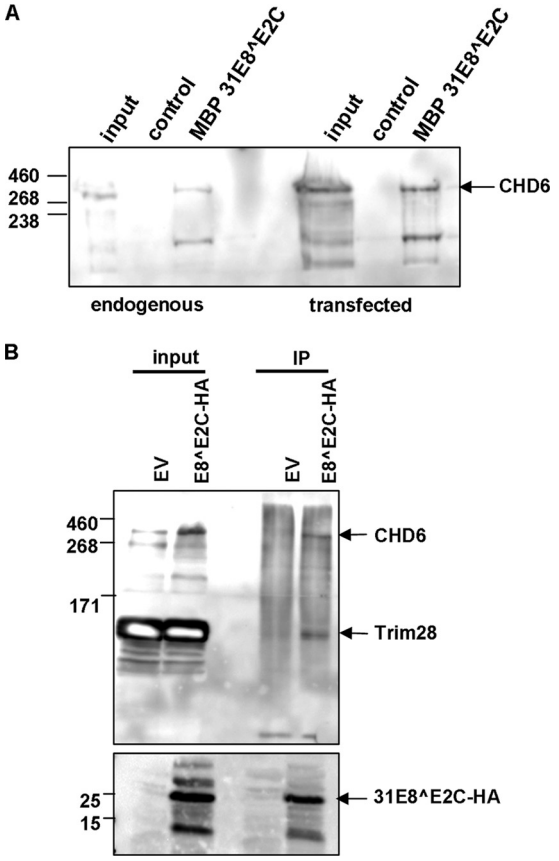

FIG. 3. (A) 31E8^E2C interacts with full-length CHD6. The empty vector (left panel) or pcDNA5.1 FR/TO CHD6 was transfected in HeLa cells (right panel), and 48 h posttransfection lysates were prepared. Similar amounts of bacterially expressed and purified MBP as a control (control) and MBP-31E8^E2C (MBP 31E8^E2C) were incubated with these cell extracts. After washing several times, bound proteins were analyzed by immunoblotting for the presence of CHD6 with a CHD6-specific antibody. (B) 31E8^E2C-HA interacts with CHD6 *in vivo.* 293T cells were stably transfected with pIRES puro (empty vector [EV]) or pIRESpuro-31E8^E2C-HA (E8^E2C-HA). Lysates were prepared and incubated with HA antibody coupled to magnetic beads, and retained proteins were analyzed by immunoblotting for the presence of CHD6 or for the presence of Trim28 (upper panel). The presence of HA-tagged protein and the efficiency of the immunoprecipitation (IP) were examined by Western blotting using an antibody against HA (lower panel).

full-length CHD6. To further confirm that this band corresponds to CHD6, HeLa cells were transiently transfected with an expression vector encoding the full-length cDNA of CHD6. The antibody recognized an identical set of bands, which were more abundant in the presence of the CHD6 expression vector (input lanes endogenous versus transfected). Both the endogenous and the overexpressed CHD6 protein interacted specifically with MBP-31E8^E2C, but not with MBP alone (Fig. 3A). This was further confirmed by coimmunoprecipitation assays using 293T cells that stably expressed 31E8^E2C-HA. Whole-cell lysates were precipitated with an HA-antibody and then analyzed by immunoblotting for the presence of CHD6 (Fig. 3B). Only immunoprecipitates from cell lines expressing 31E8^E2C-HA showed specific signals for CHD6 and TRIM28 (KAP-1), a previously described interaction partner

of 31E8^E2C (1). Taken together, by using different constructs and different experimental approaches, we demonstrated that there is a specific interaction between full-length CHD6 and $E8_{\wedge}E2C$.

31E8^E2C mutant proteins with reduced binding to full-length CHD6 display reduced transcriptional repression activity. To elucidate the functional aspects of the 31E8^E2C/CHD6 interaction, we attempted to identify mutations in the E2C domain that reduced binding to CHD6. Based on the published HPV31 E2C structure, amino acids 159, 162, 167, and 181 were selected, as they are surface-exposed residues distant from the DNA binding surface and the dimerization interface (see Fig. S1 in the supplemental material) (8, 33). In addition, we mutated residues K118 and C119, which mediate sequence-specific DNA binding (64). The mutant proteins were expressed as MBP-31E8^E2C fusion proteins and purified from bacteria. Equal amounts of fusion proteins were incubated with cell extracts and tested for the retention of full-length CHD6 by immunoblotting (Fig. 4A). After normalization to the MBP-fusion protein input, binding efficiencies were determined. This revealed that the exchange of residue N167 to alanine reduced binding to full-length CHD6 approximately 3-fold compared to the wild-type protein (Fig. 4A). In addition, an ~2-fold reduction was observed when R162 was mutated (Fig. 4A). In contrast, the exchange of residues 118/119, 159, or 181 did not influence binding of CHD6 to 31E8^E2C (Fig. 4A). To analyze the effects of the mutants on transcription, transient-transfection assays with two different 31E8^E2C-responsive promoter constructs were carried out in HeLa cells. In plasmid pGL18URR-luc, luciferase expression is driven by the HPV18 E6/E7 promoter and plasmid pC18-SP1-luc harbors four E2BS upstream of a minimal promoter. Cotransfection of wild-type 31E8^E2C-expressing plasmids with either pC18-SP1-luc or pGL18URR-luc reduced luciferase activity by 40-fold and 60-fold, respectively (Fig. 4B). Mutation of T159 or Y181 displayed repression levels comparable to the wild type. In contrast, both N167A and R162A showed ~4-fold and ~10-fold higher luciferase levels with pC18-SP1-luc and pGL18URR-luc, respectively, compared to the wild-type 31E8^E2C protein, which correlated with the loss of binding to CHD6 (Fig. 4A). Similar results were also obtained in the HPV-negative keratinocyte cell line RTS3b. Both the N167A and R162A mutants were significantly impaired in the repression of HPV18 URR activity (Fig. 4C). Immunoblotting analyses demonstrated that all mutant proteins were expressed in similar amounts (Fig. 4B). Next, the wild-type and mutants proteins were tested for DNA binding in pull-down assays. MBP-31E8^E2C fusion proteins were purified from bacteria and then incubated with a ^{32}P-labeled oligonucleotide containing high-affinity E2 binding site 4 derived from HPV31. After extensive washing the amount of retained DNA was determined by scintillation counting. MPB-31E8^E2C retained significantly more oligonucleotide than MBP alone, $31E8^{\wedge}E2C_{1\text{-}91}$, which lacks the DNA binding domain, or 31E8^E2C mutant 118/119, in which the DNA recognition helix is mutated (Fig. 4D). In contrast, mutants T159A, R162A, and Y181A bound identically to the wild type (Fig. 4D). Mutant N167A had slightly reduced DNA binding activity (Fig. 4D). This strongly suggests that the reduced repression activities of R162A and N167A are mainly due to a decreased binding to CHD6 and not due to reduced binding to DNA.

CHD6 knockdown modulates the 31E8^E2C-mediated repression of the endogenous E6/E7 promoter. To further investigate the functional consequences of the E8^E2C-CHD6 interaction, HeLa cell lines were established in which the amounts of CHD6 were reduced by lentiviral expression of two different shRNAs directed against CHD6 (shCHD6-1 and shCHD6-2). Immunoblot analyses of whole-cell lysates of drug-selected pooled cultures revealed that in both cell lines CHD6 protein expression was reduced to approximately 35% compared to the control shRNA (Fig. 5A). No severe defects in morphology or growth could be observed. The expression of 31E8^E2C in HPV18-positive HeLa cells leads to growth inhibition via the repression of the endogenous, integrated HPV18 E6/E7 promoter, similar to what has been described for papillomavirus E2 proteins (13, 25, 43, 64). This effect can be quantified by colony formation assays. HeLa-shcontrol, HeLa-shCHD6-1, and HeLa-shCHD6-2 cells were transfected with either the pIRES-neo (empty vector) or pIRES-neo-31E8^E2C-HA plasmids, from which 31E8^E2C-HA is expressed together with the neomycin resistance gene from a bicistronic RNA, ensuring a strict correlation between transgene expression and drug resistance. Cells were grown for 12 days in the presence of neomycin, and then drug-resistant colonies were counted. In HeLa-shCHD6-1, HeLa-shCHD6-1,and HeLa-shControl cell lines similar colony numbers were obtained with the empty vector, indicating that the knockdown of CHD6 had no effect on the formation of drug-resistant colonies *per se* (Fig. 5B). Expression of 31E8^E2C gave rise to 1.4 colonies on average in the HeLa-shcontrol cell line (Fig. 5B). In contrast, colony formation by 31E8^E2C increased to 26.8 and 11 in HeLa-shCHD6-1 and HeLa-shCHD6-2 cell lines, respectively (Fig. 5B). Western blot experiments confirmed that the amount of 31E8^E2C is not influenced by the knockdown of CHD6, and thus the increased colony formation is not due to reduced 31E8^E2C protein levels (Fig. 5B). To directly confirm that repression of the HPV18 E6/E7 promoter by E8^E2C is modulated by knockdown of CHD6, the HPV18 E6/E7 reporter plasmid pGL18URR-luc was cotransfected with the empty vector pSG5 or with the HPV31 E8^E2C expression plasmid in HeLa-shCHD6-1, HeLa-shCHD6-1, and HeLa-shControl cell lines. This revealed that repression of the HPV18 URR by E8^E2C is significantly impaired by a reduction of CHD6 levels (Fig. 5C). Taken together, these data strongly suggest that the interaction of CHD6 with E8^E2C contributes to the repression of the endogenous HPV18 E6/E7 promoter.

Binding to CHD6 modulates repression but not activation by 31E2. To investigate whether CHD6 binding also influences transcriptional activities by E2, the mutations described for 31E8^E2C (Fig. 4) were introduced in the 31E2 protein, resulting in T347A, R350A, N355A, and Y369A. Western blot analysis of HA-tagged proteins revealed that all mutants were expressed at amounts similar to the wild-type protein (Fig. 6A). Transient-reporter assays were carried out with plasmids pC18-SP1-luc and pGL18URR-luc and the different 31E2 mutant proteins in HeLa cells. All mutants displayed an identical

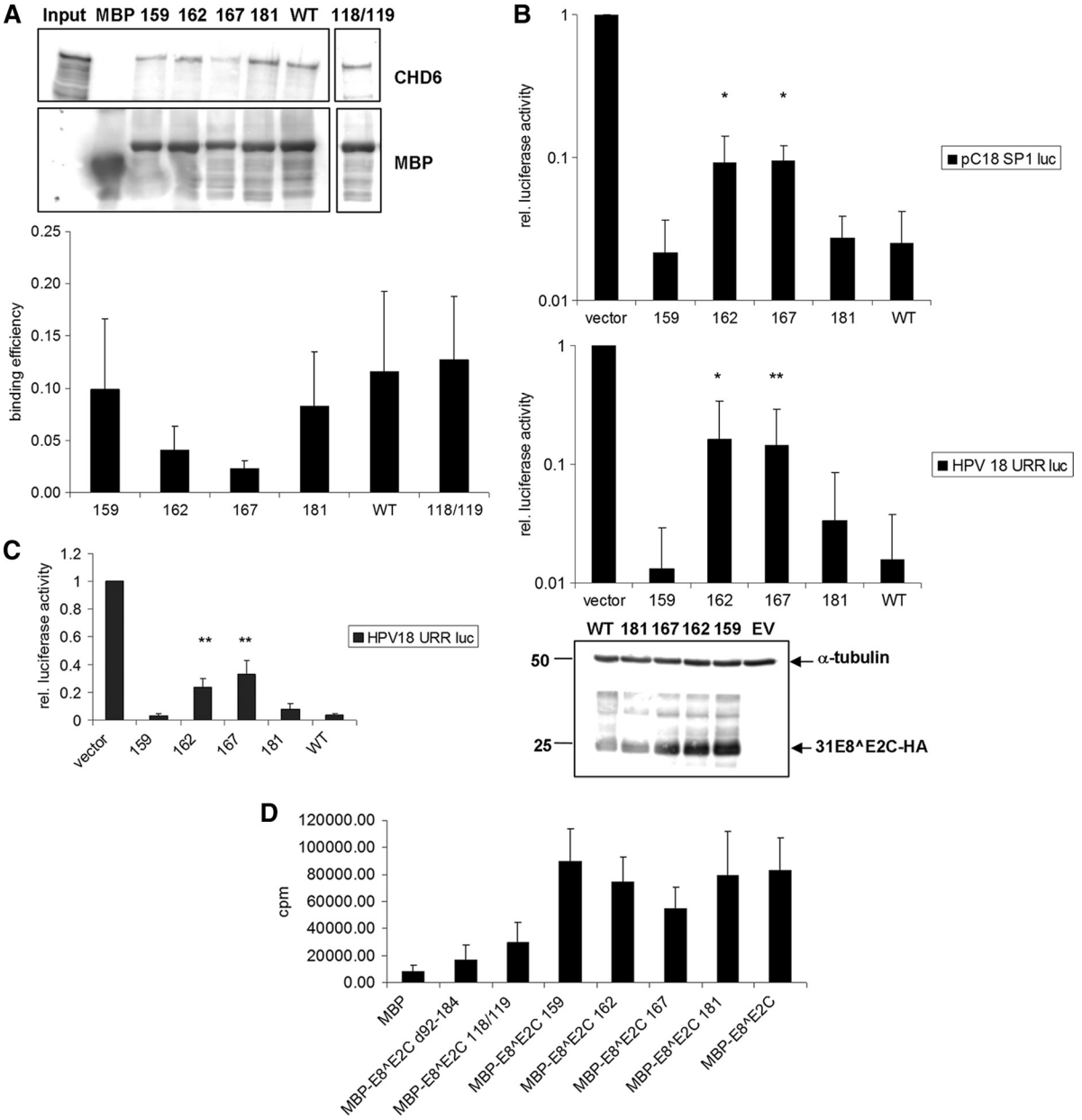

FIG. 4. (A) Single point mutations in 31E8^E2C result in reduced binding efficiency to CHD6. Upper panel: bacterially expressed and purified MBP, MBP-31E8^E2C, and the MBP-31E8^E2C-fusion proteins with the indicated single point mutations were incubated with whole-cell extracts derived from HeLa cells transfected with pcDNA5.1 FR/TO CHD6 to enhance CHD6 expression. Beads were washed several times, and retained proteins were analyzed by immunoblotting for the presence of CHD6 or MBP (lower panel) as a loading control. In the input lane, a 5% aliquot of the cell extract is shown. One representative Western blot of three is shown. Lower panel: quantification of bound CHD proteins using the QuantityOne software normalized to MBP. Data are presented relative to the input. Data represent the averages of three independent experiments. Error bars indicate the standard deviations. (B) Single point mutations in 31E8^E2C result in reduced repression activity in HeLa cells. Upper panel: HeLa cells were transfected with 50 ng of pGL18URR-luc (HPV18 URR luc) or 50 ng of pC18-SP1-luc and 10 ng of expression plasmids as indicated, and luciferase activities were determined 48 h after transfection. The data represent the averages of at least four independent transfections performed in duplicate. Asterisks indicate statistical significance levels (*, $P < 0.05$; **, $P < 0.01$) as determined by Mann-Whitney U-test. Luciferase activities are relative to the activity of pGL18URR-luc or pC18-SP1-luc in the presence of pSG5 (vector), which was set to 1. Error bars indicate the standard deviations. Lower panel: HeLa cells were transfected with 1 μg of the empty vector pSG5 (EV), 31E8^E2C-HA, or the respective mutant. Whole-cell lysates were prepared and analyzed by immunoblotting with an anti-HA antibody and anti-α-tubulin as a loading control. (C) Single point mutations in 31E8^E2C result in reduced repression activity in HPV-negative RTS3b cells. Cells were transfected with 50 ng of pGL18URR-luc (HPV18 URR luc) and 10 ng of expression plasmid as indicated, and luciferase activities were determined 48 h after transfection. The data represent the averages of at least two independent transfections performed in triplicate. Asterisks indicate the statistical significance level (**, $P < 0.01$) as determined by Mann-Whitney U-test. Luciferase activities are relative to the activity of pGL18URR-luc (HPV18 URR luc) in the presence of pSG5 (vector), which was set to 1. Error bars indicate the standard deviations. (D) Single point mutations in 31E8^E2C do not impair the DNA binding ability. Bacterially expressed and purified MBP, MBP-31E8^E2C, or the MBP-31E8^E2C-fusion proteins of the indicated mutants were incubated with ^{32}P-labeled double-stranded oligonucleotide matching the E2 binding site aa 45 to 70. Beads were washed, and bound oligo was measured using a scintillation counter. The *y* axis represents the cpm, and the average of seven independent experiments is shown. Error bars indicate the standard deviations.

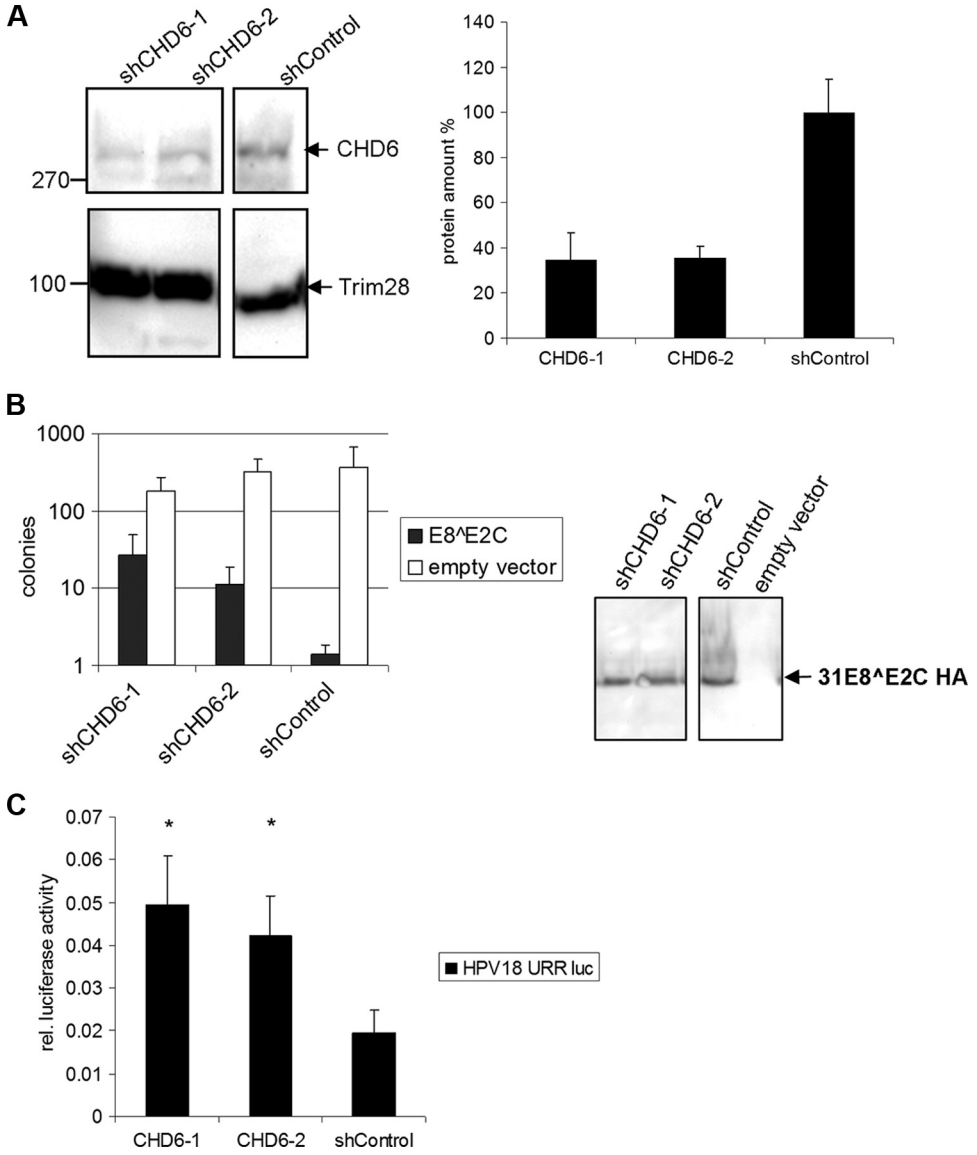

FIG. 5. (A) shRNAs directed against CHD6 decrease CHD6 protein levels in HeLa cells. HeLa cells were infected with lentiviral particles expressing two different shRNAs against CHD6 (shCHD6-1 and shCHD6-2) or a control shRNA (shControl) and then selected for 14 days with puromycin. Cell pools were tested by Western blotting for reduced CHD6 protein amount. Whole-cell lysates were prepared and analyzed with an anti-CHD6 antibody and an antibody against Trim28 as a loading control. On the left a molecular size marker (in kDa) is indicated. One representative Western blot is shown. Quantification of CHD protein levels using the QuantityOne software (Bio-Rad) is shown in the right panel. Data presented are relative CHD6 protein levels compared to the shControl-infected cells. (B) Decreased CHD6 levels result in an increased colony number formation by 31E8^E2C in HeLa cells. Left panel: colony formation assay. HeLa cells stably infected with two different shRNAs against CHD6 (shCHD6-1 and shCHD6-2) were transfected with a neomycin resistance plasmid expressing 31E8^E2C-HA (pIRESneo-31E8^E2C-HA) or the empty vector. The graph represents the averages of the neomycin-resistant colonies derived from five independent experiments. Standard deviations are indicated by error bars. Right panel: Western blot analysis of whole-cell lysates obtained from HeLa cells carrying either the shRNA against CHD6 (shCHD6-1 and shCHD6-2) or shControl (shC) transfected with either pIRESneo-31E8^E2C-HA or empty vector (pIRESneo) using anti-HA antibody. (C) HeLa cells expressing shRNAs directed against CHD6 (CHD6-1 and CHD6-2) or a control shRNA (shControl) were transfected with 50 ng of pGL18URR-luc (HPV18 URR luc) and 10 ng of 31E8^E2C expression plasmid, and luciferase activities were determined 48 h after transfection. Luciferase activities are relative to the activity of pGL18URR-luc (HPV18 URR luc) in the presence of empty vector, which was set to 1. Error bars indicate the standard errors. The data represent the averages of at five independent transfections performed in duplicate; *, $P < 0.05$ as determined by Student's t test.

(90-fold) but slightly weaker activation than the wild-type 31E2 protein (140-fold), which was statistically not significant (Fig. 6B). Since the mutants differ in their binding to CHD6 (Fig. 4), this suggests that CHD6 does not contribute to transactivation by 31E2. Promoter activity of pGL18URR-luc was repressed by wild-type 31E2 to 10% of the basal activity (Fig. 6C). Mutation of R350A or N355A resulted in a statistically significant 3-fold increase in reporter gene activity, whereas T347A and Y369A behaved identical to wild-type 31E2 (Fig. 6C). As found with 31E8^E2C, only mutations that impaired binding

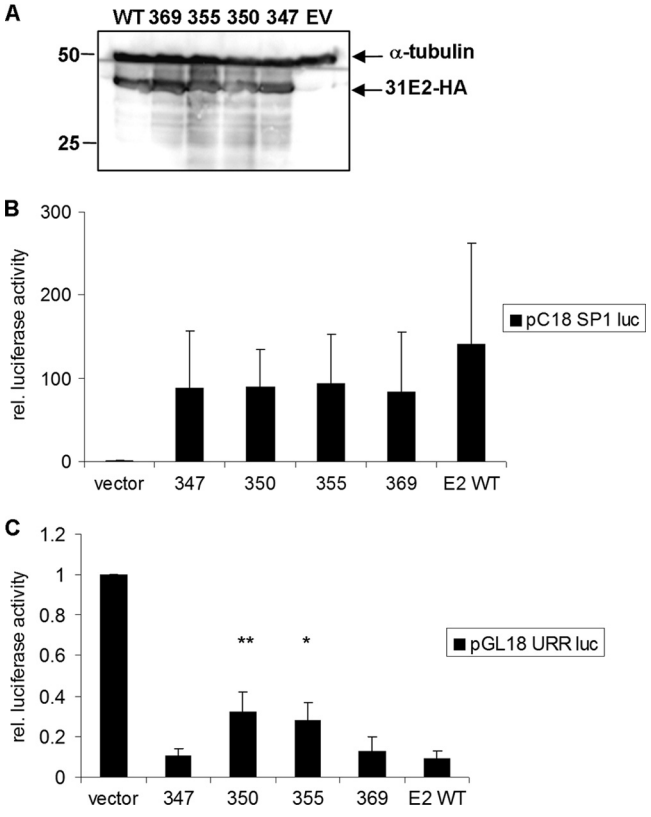

FIG. 6. Single point mutations in 31E2 result in reduced repression activity in HeLa cells. (A) HeLa cells were transfected with 1 μg of empty vector pSG5 (EV), expression plasmids for 31E2-HA (WT), or the respective mutants (347, 350, 355, and 369). Whole-cell lysates were prepared and analyzed by immunoblotting using an anti-HA antibody and anti-α-tubulin as a loading control. (B) HeLa cells were transfected with 50 ng of pC18 SP1 luc and 10 ng of the indicated expression plasmids, and luciferase activitiy was determined 48 h post-transfection. The data represent the averages of five independent transfections performed in duplicate. Luciferase activities are relative to the activity of pC18 SP1 luc in the presence of pSG5 (vector), which was set to 1. Error bars indicate the standard deviations. (C) HeLa cells were transfected with 50 ng of pGL18 URR luc and 10 ng of expression plasmid for 31E2 or 31E2 mutants. Luciferase activity was determined 48 h after transfection. The data represent the averages of six independent transfections performed in duplicate. Asterisks indicate statistical significance levels (*, $P < 0.05$; **, $P < 0.01$) as determined by Mann-Whitney U-test. Luciferase activities are relative to the activity of pGL18 URR luc in the presence of pSG5 (vector), which was set to 1. Error bars indicate the standard deviations.

to CHD6 displayed a reduced repression of the E6/E7 promoter. This strongly suggests that CHD6 is involved in transcriptional repression by both 31E8^E2C and 31E2.

DISCUSSION

Several human and animal papillomaviruses generate both the full-length E2 protein and the E8^E2C protein (E9^E2C for CRPV) from the E2 gene (9, 18, 28, 46, 52, 58, 63). Both proteins share the hinge region and the E2C part which is responsible for sequence-specific DNA binding and the dimerization of E2 proteins, but they differ in their amino-terminal domains (38). The only known common function of both proteins is the transcriptional repression of the HPV E6/E7 promoter (3, 6, 31, 63-65). Transcriptional repression by E2 and E8^E2C is absolutely dependent upon the presence of E2BS elements in the E6/E7 promoter and requires the E2C domain (17, 24, 51, 65, 68, 71).

In this study we demonstrated that the 31E2C domain not only contributes to repression via sequence-specific DNA binding but also via a conserved interaction with the cellular CHD6 protein. Both RNA interference knockdown and mutational analyses suggest that this interaction contributes to the transcriptional repression activity by 31E8^E2C and 31E2.

CHD6 acts as a coactivator for the cellular Nrf2 transcription factor, whereas our data suggest a corepressor role for 31E8^E2C (42). Dual roles for CHD proteins as both activators and repressors have been described for many family members. CHD3 and -4 have been identified as components of NURD complexes, which are involved in transcriptional repression (78, 84). However, CHD3 can also be a coactivator for the c-myb transcription factor (53). Similarily, CHD8 is a repressor of HOXA2 transcription and of beta-catenin target genes and acts as a corepressor for p53 during early mouse embryogenesis (44, 72, 80), but CHD8 is also an activator of the cyclin E2 gene (50). CHD7 is part of a corepressor complex bound by peroxisome proliferator-activated receptor γ (PPAR-γ) (67) but acts also as a transcriptional activator of Sox9, Twist, and Slug transcription to induce the neural crest transcriptional circuitry (2).

Repression activity of the NURD complex has been ascribed to the ATP-dependent chromatin remodeling activities of CHD3 and -4 and to histone deacetylation by associated histone deacetylases (HDACs) 1 and 2 (15). CHD8 has been reported to recruit a WDR5/Ash2l/RBBP5 complex to inhibit HOXA2 and beta-catenin targets (72, 80), whereas p53-dependent transcription is inhibited by a trimeric p53/CHD8/histone 1 complex (44). PPAR-γ-dependent transcription is inhibited by a CHD7/SETDB1/NLK complex (67). Taken together, these findings indicate that CHD proteins are part of different multiprotein complexes to repress transcription. CHD6 has been described to be a component of a 2- to 3-MDa complex in unstimulated HeLa cells, but the identity of these components remains to be identified (34). It is possible that E8^E2C recruits via CHD6 such a multimeric complex that has transcription repression activity. Alternatively, E8^E2C may block an activating function of CHD6.

The contribution of the E2C-CHD6 interaction to the repression activity of 31E8^E2C appears smaller than the interaction of the 31E8 domain with the HDAC3/NCor1 complex (1, 48, 64). One explanation is that CHD6 protein levels were only reduced to ~35% by shRNA knockdown and that the remaining CHD6 is sufficient for some repression activity. Similarly, 31E8^E2C mutants still retained some binding activity, which may lead to an underestimation of the CHD6 contribution to repression activity. It has been described that the formation of some CHD-containing complexes is dependent on the presence of extracellular ligands. Formation of the CHD7/NLK/SETDB1 repressor complex occurs only in the presence of Wnt5a (67). Thus, it is possible that repressive activity of CHD6 or the E2C/CHD6 interaction may be influenced by extracellular ligands allowing for a fine-tuning of the HPV E6/E7 promoter activity. Interestingly, CHD1 has been found

to repress transcription from latent HIV-1 genomes, which prevents HIV reactivation (73). Thus, CHD6 might play a modulatory role during persistent HPV infections *in vivo*.

Immunoprecipitates of CHD1 have been reported to display HDAC activity and CHD1 associates with NCor1 in yeast two-hybrid and *in vitro* pull-down assays (66). However, biochemically purified NCor1 complexes do not contain CHD6 or other CHD family members (81, 83). Furthermore, the 31E8 domain has been demonstrated to be a transferable repression domain (85). This suggests that CHD6 functions independently from the HDAC3/NCor1 complex and that E2C acts as a second independent repression domain for the URR. This is consistent with the finding that the E2C part derived from HPV11, -16, -18, or 31 has significant repression activity on its own in transient-transfection experiments using HPV URR promoter constructs (11, 13, 17, 65). It was also demonstrated that E2C of HPV11 is able to repress the URR in an *in vitro* transcription system comparably to E2 (24). This phenomenon has been attributed to binding site competition between E2 and cellular transcription factors such as SP1 and TATA box binding protein (14, 17, 19, 69). Since these competition and *in vitro* transcription experiments were performed in the presence of nuclear extracts or immunopurified components from HeLa cells, it is possible that some CHD6 was present and contributed to repression.

The HPV8 E2 protein has been demonstrated to bind to and repress transcription of the cellular ITGB4 gene (45). Interestingly, repression was also observed when only the E2C domain of HPV8 was used (45). Due to the high conservation of the E2C DNA binding domain among papillomaviruses, it is possible that CHD6 also interacts with HPV8 E2C and contributes to repression of the ITGB4 gene. This raises the possibility that the expression of putative CHD6 cellular targets might be altered by E8^E2C and/or E2.

Taken together our results indicate that the E2C domain not only mediates specific DNA binding but also has a role in transcriptional repression by recruitment of the CHD6 protein. This suggests that repression of the E6/E7 promoter by E2 and E8^E2C involves multiple interactions with host cell proteins that are mediated by different protein domains.

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